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Template version 09/11

TXR#: 0057772

DATA EVALUATION RECORD

STUDY TYPE: Special Study – Assessment of Acoustic Startle Response in Juvenile and Adult Rats Following a Single Oral Dose of Permethrin.

PC CODE: 109701 **DP BARCODE:** D444188

TEST MATERIAL (PURITY): Permethrin (95.6%)

SYNONYMS: (3-phenoxyphenyl)methyl 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate

CITATION: Vorhees, C., and Williams., M. (2016) Assessment of Acoustic Startle Response in Juvenile Rats Following a Single Oral Dose of Permethrin. Cincinnati Children's Research Foundation Div. of Neurology Neuroscience Laboratory. Laboratory ID: Permethrin Rat Juvenile-Adult Neurotoxicity Study. May 23, 2016. MRID 50409302 Unpublished.

SPONSOR: Council for the Advancement of Pyrethroid Human Risk Assessment, LLC (CAPHRA) C/o Consumer Specialty Products Association

EXECUTIVE SUMMARY: Several preliminary studies (summarized below) were performed to establish the suitability of acoustic startle response (ASR) to measure acute neurotoxicity effects in adult and juvenile rats and to establish doses for the definitive studies. In the definitive studies, permethrin was administered to both adult and PND15 rats at doses of 0, 60, 90, or 120 mg/kg in 5 mL/kg of corn oil. Juveniles (16/sex/group, 1/sex/litter) were then observed for detailed clinical observations and acoustic startle responses were measured at 2, 4, 6 and 8 h after administration.

No mortality was observed in the definitive experiments. Detailed clinical observations were limited in adult rats to pre-treatment only, with limited clinical observations post-treatment. At 90 mg/kg/day, 2 animals were reported with increased salivation after 4 h. At 120 mg/kg/day, 1 animal was reported with increased salivation after 4 h, and 3 animals were reported to have tremors following 6 and 8 h. At 6-h post exposure, considered to be the time to peak effect for adult animals, ASR increased 3.4-fold and 3-fold at the mid- and high-doses, respectively.

Significant clinical signs were observed in pups. At 60 mg/kg, mild (9/16 females; 7/16 males) to moderate (5/16 females; 7/16 males) effects in motility and mild (11/16 females; 10/16 males) to moderate (0/16 females; 1/16 males) tremors were observed from 2 to 8 h post exposure. At 90 mg/kg, moderate (6/16 females and males) to severe (10/16 females and males) effects in motility and moderate (3/16 females; 2/16 males) to severe (13/16 females; 14/16 males) tremors were observed with an apparent time to peak effect of around 4 to 6 h post exposure. At 120

mg/kg, severe effects in motility (15/15 females, 13/15 males) and severe tremors (15/15 females; 16/16 males) were observed with an apparent time to peak effect of around 4 to 6 h post exposure. It is important to note the scoring system used did not differentiate between hyper- or hypo-active individual; therefore, the motility scores do not provide a good indication of difference in activity. A dose-dependent increase in ASR was only observed at 2-h following administration for pups. Treatment with permethrin increased the ASR in PND15 rats.

Although PND15 pups appeared to be more sensitive than adults to the neurotoxic effects of permethrin based on clinical observations, the available data did not demonstrate an appropriate magnitude or time-course of effects on acoustic startle in PND15 rats. In both preliminary and definitive studies, the available data indicate that tremors interfered or confounded the results of the acoustic startle testing. Due to this, appropriate doses for PND15 pups were not established, which in turn did not allow study authors to establish a clear time-to-peak effect, or post-treatment interval for testing Therefore, a quantitative comparison between juveniles and adults is not appropriate using ASR data in this study.

The LOAEL for pups is 60 mg/kg, based on mild decreases in motility and mild to moderate tremors observed from 2 to 8 h post exposure. The NOAEL is not established.

The LOAEL for adults is 60 mg/kg, based on increased ASR observed from 2 to 8 h post exposure. The NOAEL is not established.

I. MATERIALS AND METHODS:

A. MATERIALS:

1. Test material: 40:60 Permethrin Technical

Description:	Clear Liquid
Lot/batch #:	VTS-1066-33A
Purity:	95.6 % a.i.
CAS # of TGAI:	52645-53-1

2. Vehicle and/or positive control: Corn Oil 5 mL/kg (unless otherwise specified)

3. Test animals:

Species:	Rat	
Strain:	Sprague-Dawley (SD)	
Age/weight at dosing:	12-13 Weeks in adult studies PND 15 to 17 in Juvenile studies	
Source:	Charles River Laboratories, Raleigh, NC	
Housing:	2 Rats/cage	
Diet:	Guaranteed Analysis NIH-007 Rodent Diet from PMI Corp, *ad libitum*	
Water:	Deionized and reverse osmosis-filtered, *ad libitum*	
Environmental conditions:	**Temperature:**	19 ± 2°C
	Humidity:	50 ± 20%
	Air changes:	30/h
	Photoperiod:	14 h light/10 h dark
Acclimation period:	3-4 Weeks	

B. PRELIMINARY/RANGE-FINDING STUDIES

1. Adult Preliminary Studies:

Study 1:

Objective: The objective of this experiment was to test the effects of single oral doses of permethrin in adult rat acoustic startle peak response amplitude 2 h post-treatment.

Methods: Permethrin was dissolved in the vehicle (corn oil) and administered by gavage as a single dose to each group (Groups 1, 2, 3, and 4, respectively) of Sprague-Dawley male rats (Charles River, strain 001, Raleigh, NC). Dose levels were 0, 60, 90, and 120 mg/kg of permethrin. The control group (Group 1) received the vehicle in a comparable regimen in the same dosing volume of reagent grade corn oil (Sigma-Aldrich, St. Louis, MO), i.e., in a volume of 5 mL/kg. At the time of dose administration, adult males were approximately 9 weeks old. Animals were observed daily for health or signs of illness. Clinical examinations and body weight measurements were performed prior to and on the day of dose administration. ASR evaluations began 2 h after dose administration, which was 2 h after removal from access to food (i.e., startle testing began 4 h post-food removal in adults but not in PND15 rats). Following ASR, all rats were euthanized and discarded in accordance with standard laboratory procedures.

Results/Investigator's Conclusions: Each test session began with a 5 min acclimation period in the test apparatus, prior to the onset of the first acoustic stimulus. Following the 5 min no-stimulus acclimation period, 100 acoustic startle stimulus trials were presented with 20 s inter-trial intervals. Data were organized into 10 trial blocks. Data were analyzed separately by hour by mixed linear ANOVA with factors of Treatment x Block and resulting in a 1-between (4 dose level factor) x 1-within (10 block factor) fixed effect factorial ANOVA model. Data were analyzed using SAS statistical applications (SAS Institute, v9.3, Cary, NC).

All treated adult male rats survived treatment. Two hours following dose administration, no clinical observations of salivation, tremor, or abnormal motor movements were observed. No evidence of writhing or vocalization was noted. There was no significant effect of permethrin groups on acoustic startle response at any dose.

Study 2:

Objective: The objective of this experiment was to evaluate ASR in untreated Sprague-Dawley male rats and assess ASR variability and whether body weight is the best method of matching animals for group assignment.

Methods: In this experiment, 12 adult SD male untreated rats were used. Each animal was tested three times on three separate days. The rats were given 100 ASR trials using the same protocol as above. The rats were tested 3 times, with 2 days between session-1 and session-2 and 4 days between session-2 and session-3.

Results/Investigator's Conclusions: The correlation coefficient for V_{max} for day-1 vs. day-2, was $r = 0.67$; between day-1 vs. day-3, was $r = 0.67$, and between day-2 vs. day-3, was $r = 0.83$. These correlations demonstrate that the first ASR session is more variable than subsequent sessions and less predictive of ASR on subsequent days. For this reason, in further experiments rats were acclimated by pretesting them for acoustic startle before testing with permethrin.

To determine the relationship between body weight and acoustic startle, correlational analyses were conducted on the matched body weights by group from the main experiment above (Adult – ASR at 2 h post-treatment) to the 2 h ASR data. The correlation between body weight and ASR following treatment with permethrin was $r = -0.17$, which did not differ significantly from zero. These data demonstrate that body weight has a slight and negative relationship to V_{max} and showed that body weight is not well-suited for matching groups for acoustic startle assessment.

Study 3:

Objective: The purpose of this experiment was to see if there are strain differences in the acoustic startle of rats given the same dose of permethrin in the dose volume used by Crofton et al., i.e., 1 mL/kg. Long-Evans (LE) and Sprague-Dawley (SD) rats were used.

Methods: Animals for this experiment were 201-225 g Long-Evans male rats from Charles River, Raleigh, NC and 201-225 g Sprague-Dawley from Charles River, Raleigh, NC. Group sizes were 6 rats per strain per group. Rats were treated with corn oil alone or corn oil containing a 120 mg/kg dose of permethrin. The dose volume was 1 mL/kg given by gavage. Body weights were measured prior to treatment and rats were observed for detailed clinical observations.

Rats received the following procedures prior to permethrin treatment:

- Week 1: Rats arrived on Tuesday and weighed every other day (Wednesday, Friday, Sunday)
- Week 2: Rats received 4 trials in a 244 cm straight swimming channel twice (Tuesday and Thursday). Next, they received a single ASR session on Friday (no gavage).
- Week 3: Rats received corn oil by gavage with no test article added and given a single acoustic startle response session (Monday.); Tuesday and Thursday rats were ASR tested in a crossover design in which half received vehicle and half received permethrin on Tuesday and those that received vehicle on Tuesday received permethrin on Thursday.

On the treatment days, rats were tested 2 and 4 h after treatment. Food was removed 2 h prior to treatment.

Results/Investigator’s Conclusions: (Summary Tables in Appendix 1, Figures A1 and A2) The main effect of treatment and the treatment x time interaction were significant. Hence, the permethrin-treated Long Evans rats showed significantly increased ASR at 2 and 4 h post-treatment. This experiment changed two variables simultaneously, i.e., dose volume and pretest experience, therefore, the independent effects of each cannot be determined. Nevertheless, the net effect of both changes together indicate that Long Evans rats show a robust ASR increase after 120 mg/kg of permethrin that begins by 2 h post-treatment and remains increased at 4 h post-treatment. Permethrin in SD treated rats also showed increased ASR across test times at 2 and 4 h post-treatment. This experiment differed from Experiment-1 in two ways: 1) a dose volume of 1 mL/kg was used and 2) the animals had ASR pretesting, as in the previous preliminary experiment with Long Evans rats. Together the data indicate that SD rats show a robust acoustic startle increase after 120 mg/kg in 1 mL/kg corn oil of permethrin that begins by 2 h post-treatment and remains at 4 h post-treatment.

The results of these two preliminary experiments in Long Evans rats treated with permethrin in 1 mL/kg of corn oil replicated the findings reported by Crofton and Sheets. Accordingly, the central issue in the replication inconsistency in the first adult preliminary permethrin experiment was caused by the difference in dose volume rather than rat strain. This is evidenced by the fact that both strains showed robust ASR increases and did so at both times of testing after treatment. The initial experiment conducted with permethrin in 5 mL/kg corn oil and tested 2 h post-treatment did not rule out that the effect may have had a delayed onset because of the larger dosing volume such that an effect may have present by its manifestation delayed by a longer time of absorption.

Study 4 & 5:

Objective: Determine time course of effects for permethrin administered in 5 mL/kg of corn oil.

Methods: Groups of 6 SD rats per group were administered 120 mg/kg of permethrin in 5 mL/kg of corn oil. ASR was measured at 2, 4, and 6 h post-dosing. In a second study an 8 h time point was included.

Results/Investigator’s Conclusions: (Summary Tables in Appendix A, Tables A3 and A4) Permethrin significantly increased ASR at 4 h post-treatment and produced a non-significant trend at 6 h, which is consistent with the literature showing that permethrin increases ASR as it did in pilot experiments when the test article was given in a dose volume of 1 mL/kg. The main effect of treatment was significant ($p < 0.0011$). The treatment x block interaction approached significance ($p = 0.052$). Given 6 rats per group, the fact that the treatment x block interaction was nearly significant suggests that time-dependent effects of permethrin can be predicted to be significant with a larger sample size.

Permethrin significantly increases ASR (main effect) in adult SD rats and this effect is time-dependent with peak effect at approximately 6 h post-treatment (Figure 1). Based on the results of the adult permethrin study above and the four follow-up preliminary experiments, the data support that doses of permethrin of 0, 60, 90, and 120 mg/kg were appropriate but that other factors (dose volume and time-course) were off target in the initial experiment. The source of non-replication between the literature and the initial experiment was not because of the strain of rat, or the apparatus, but was attributable to the larger dose volume (5 mL/kg) delaying the onset of the ASR change compared with what had been used in the literature (0.2-1 mL/kg). The change in dose volume shifted the time-course for absorption of the test article, causing the effects to be delayed beyond 2 h; intervals that were not tested in the first experiment.

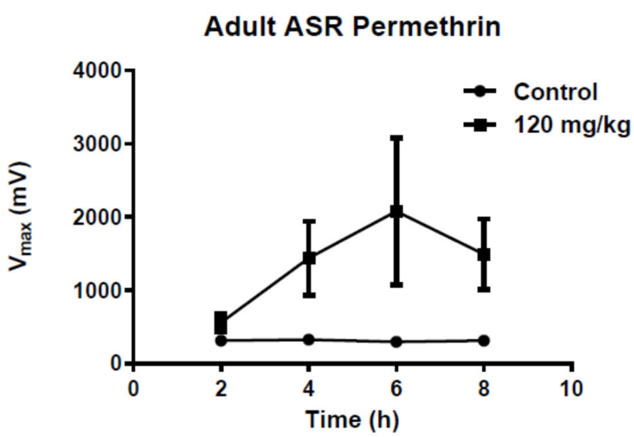

Figure 1. Effects of permethrin on ASR in adult male rats as a function of time since treatment. ASR was tested for 100 trials at each time. (MRID 50409302; Figure 3, page 38).

Therefore, based on this interpretation, the definitive adult permethrin experiment should be conducted using the same doses (0, 60, 90, and 120 mg/kg) in 5 mL/kg with repeated ASR testing at 2, 4, 6, and 8 h post-treatment. In addition, based on the ASR-1 study that matching groups based on ASR pretesting improves the matching of groups compared with matching based on body weight, the definitive adult permethrin study again used this procedure to balance groups for startle reactivity.

EPA Reviewer Comments on Adult Preliminary Data: The reviewer agrees with the general conclusion that treatment with permethrin resulted in significantly increased ASR in male SD rats. The reviewer also agrees that there were no apparent strain differences or significant impacts due to the difference in apparatus used.

Additionally, the reviewer agrees with matchmaking of groups based on ASR pretesting versus bodyweight. The reviewer agrees that the difference between the literature values and the initial testing was due to dose volume differences. However, the reviewer does not agree that increased dose volume delayed the onset of ASR. It is well established that increased volumes of corn oil decreased the magnitude of neurotoxic effects that result from pyrethroid treatment. The data in the 1 mL/kg corn oil studies in both strains demonstrated a high magnitude response at 4 h versus 2 h. A 6 h time point was not evaluated. However, given that the magnitude of response at 4 h with 1 mL/kg (7.3x) and at 6 h with 5 mL/kg corn oil (7.1x) were virtually identical, and that increased corn oil volume decreases pyrethroid potency, it is reasonable to conclude that a 6 h time point at 1 mL/kg would have resulted in a more significant effect than what was observed at 2 or 4 h.

It is unclear to the reviewer why 5 mL/kg was chosen as the dose volume for the definitive study, when the conclusions of the study author identified the increased dose volume as the key factor in not being able to replicate data from the literature, and when the preliminary study data demonstrated a clear impact of gavage volume on the results of the ASR test following permethrin treatment.

It is also unclear how dose selection for the definitive study was determined to be appropriate when only one of the doses selected had been investigated under proper timing and pre-test methodology.

2. Juvenile Preliminary Studies:

Objective: Assess the tolerability to permethrin during acoustic startle response testing

Method: Permethrin was administered to PND15, 17, and 21 rats at single oral gavage doses of 72, 90 and 108 mg/kg and a gavage volume of 3 mL/kg[1]. Mortality and detailed clinical observations were recorded, and ASR was measured at 2, 4, 6, and 8 h post-dosing.

Results/Investigator's Conclusions: (Summary Tables in Appendix A Tables A5-A7) Permethrin doses of 72 and 90 mg/kg on PND15, 17, or 21 was well-tolerated. Permethrin at 108 mg/kg, however, caused increased mortality at all three ages. The increase in mortality was age-dependent. The smallest increase occurred in the permethrin 108 mg/kg PND17 pups and was slightly higher in the PND15 compared with PND17 pups, and substantially increased in PND21 pups. The difference in mortality between the PND15 and PND17 pups may not be reliable because the size difference is small, and because litter differences were not accounted for in this pilot study. However, the increase in mortality in the permethrin 108 mg/kg group at PND21 approximates an LD50 and is not suitable for use in future ASR experiments.

Clinical observations results indicate that:

- Controls show no tremor;
- All PRM-treated groups at all doses (72, 90, and 108 mg/kg) and at all ages (PND15, PND17, and PND21) showed some tremor. Tremor occurred at most post-treatment times (2, 4, 6, and 8 h post-treatment);
- There was a general (non-linear) dose-dependent increase in tremor at all ages with increasing permethrin dose; the non-linearity was seen as an increase in tremor that was greater between the 72 and 90 mg/kg doses compared with that between the 90 and 108 mg/kg doses.
- There was a time-dependent change in tremor at all doses at all ages.

[1] The data from the juvenile preliminary experiments were used to determine definitive doses, but a technician made an error in administering the compound in several of the preliminary experiments. She measured out the proper amount of compound for each dose level and mixed it in the proper volume of corn oil, but when she gavaged animals she used a dosing schedule that she had transferred from another study that was based on 3 ml/kg rather than 5 mL/kg. Hence, she administered 60% of the intended dose.

At the permethrin 90 mg/kg dose, there was an increase in the incidence of tremors across time with the peak effect being age-dependent, i.e., it peaked at 4 h in the PND15 and PND17 pups, but at 2 h in PND21 pups. By contrast, at the permethrin 90 mg/kg dose, tremor peaked at 2 and 4 h at PND15, at 2, 4, and 6 h at PND17, and at 4 h at PND21. At the permethrin 108 mg/kg dose, tremor onset was rapid and was present in 100% of the pups at PND15 at 2, 4, and 6 h, nearly the same at PND17 at 2, 4, and 6 h, and exactly the same at PND21 at 2, 4, and 6 h. All treated groups showed a decline in tremor by 8 h. Overall, the data indicate that PND15 pups are the most sensitive to tremor, although the difference between PND15 and PND17 pups was modest. By contrast, the PND21 pups were substantially less sensitive to tremor induction than the younger ages.

These data indicate that PRM induces a distinct age-dependent increase in tremor and that younger rats are more sensitive than rats a few days older and much more sensitive than adults.

The results show that ASR was increased in all dose groups, ages and at most time intervals. A dose of 72 mg/kg permethrin produced a modest increase in acoustic startle response in PND17 (4 h) and PND21 (2 and 4 h) rats but did not increase it in PND15 rats by two-tailed test but did at 4 h by one-tailed test. A dose of 90 mg/kg permethrin produced an increase ASR in PND15 (2-4 h), PND17 (2-6 h), and PND21 (2-6 h) greater than at 72 mg/kg. At 108 mg/kg permethrin increased ASR but also increased mortality at all three ages. The magnitude of the ASR increase increased with age but the maximum effect (~60% above control) was less in adults (see below).

Tremor was evident at 72, 90, and 108 mg/kg, and the incidence increased with dose. More experiments are needed; however, because the data indicate that for permethrin, ASR and detailed clinical observations overlap at the doses tested in PND15, 17, and 21 rats. These preliminary data indicate that while tremor and increased ASR co-occur at all permethrin doses for all three juvenile ages, the time-course of the changes for the two effects differ. At PND15, permethrin at 72 mg/kg caused peak tremor and ASR at 4 h, whereas at PND17, tremor peaked at 4 h and ASR at 2 h. Furthermore, tremor declined by 50% by 4 h, whereas the ASR effect declined by 30% at this time; at PND21, tremor peaked at a modest incidence at 2 h and declined rapidly thereafter even as ASR increased at 4 h.

At the middle dose of 90 mg/kg permethrin, there was both concordance and discordance between tremor and acoustic startle response across time and age. At PND15, ASR effects were greatest at 2 h and declined at 4 and 6 h, whereas tremor was the same at 2 and 4 h, declined slightly at 6 h, and was small at 8 h. At PND17, ASR declined steadily from 2 to 4 h, still further from 4 to 6 h, and showed no difference at 8 h, whereas tremor was high and remained high at 2, 4, and 6 h with only a modest decline at 8 h. The largest discordance occurred at PND21 where ASR was increased at all times, whereas tremor was high at 2 and 4 h, declined to 50% at 6 h, and was no longer observed by 8 h, a time when ASR remained increased.
At the highest dose of 108 mg/kg of permethrin, discordance between tremor and ASR was greatest. At PND15, tremor was high at all times, whereas ASR was significantly increased at only 2 and 4 h. At PND17, tremor was high at 2, 4, and 6 h with a slight decline at 8 h, whereas ASR was increased at 2, 4, and 6 h and showed no effect at 8 h. At PND21, tremor was 100% at 2, 4, and 6 h and ASR was increased at these same times. The 8 h data were compromised by increased mortality.

In general, tremor and ASR increases showed overlap but the time-course of the two effects differed. At juvenile ages, it is evident that the two effects cannot be disentangled at the doses we tested because the overlap of effects was extensive; unlike in adults where the two effects are easily distinguishable. In addition, whereas in adult rats, ASR increases were dose-dependent at doses of 60, 90, and 120 mg/kg, in preweaning rats, ASR increases were only evident at doses of 72, 90, and 108 mg/kg where significant increases in tremor also occurred, and at the 108 mg/kg dose mortality emerged. Therefore, adult rats are more susceptible to ASR changes than to tremor, hence, ASR is the more sensitive indicator of effect in adults. In juvenile rats, ASR and tremor overlap at the doses tested such that ASR is as or more sensitive than tremor. However, the interpretation that ASR is more sensitive in juvenile rats than tremor must be qualified because separating the effects is at best an approximation at these doses. However, ASR is quantitative and objective whereas detailed clinical observations are neither objective nor linear; at best they are categorical.

EPA Reviewer Comments on Juvenile Preliminary Data: The reviewer disagrees with the study investigator's conclusions on mortality. The increase in mortality was not age-dependent as the PND15 juveniles had more deaths at the high-dose than PND17 juveniles. While the study author acknowledges that the difference in mortality between the PND15 and PND17 pups may not be reliable because litter was not accounted for in this pilot study, they do not acknowledge the same deficiency with respect to the PND21 pups. The toxicological relevance of all mortality findings is less clear when the litter is not accounted for. Additionally, it is difficult to reconcile the investigator's conclusion that PND15 pups are the most sensitive when the only age group with significant mortality is the PND21 pups.

The reviewer also disagrees with the study author's conclusions on tremors. The effect was described as non-linear due to the increase in magnitude being greater between the low- and mid- dose versus the mid- and high- dose. However, this is not an appropriate conclusion given that 100% of the animals in all age groups had tremors at the time-to-peak effect in the high dose. Therefore, without severity scoring it is difficult to interpret the magnitude of the increase. The reviewer also disagrees with the study investigator's conclusions on time to peak effect. Based on the weight of the evidence, the time-to-peak effect for juvenile animals in all age groups was 4 h post-exposure for tremors. At 4-h post exposure, it is difficult to differentiate PND15 and PND17 pups with respect to sensitivity. However, the reviewer agrees that PND21 pups are less sensitive to tremors compared to PND15 and 17 pups.

Permethrin did increase ASR in all age groups. However, it is difficult to determine the time-to-peak effect of acoustic startle since significant tremors were occurring at the same time. The lack of a dose response from mid- to high-dose as well as decrease in the magnitude of which acoustic startle is affected as tremors increase, suggests that the occurrence of significant tremors is confounding the acoustic startle data and dose selection for juvenile animals may be too high. Based on the preliminary data, there is a clear sensitivity to pups with respect to tremor response of permethrin. Due to the presence of increased tremors in juvenile animals and its impact on ASR, it is difficult to compare the ASR between adult and juvenile animals.

C. DEFINITIVE STUDY DESIGN:

1. **In life dates:** N/A

2. **Animal assignment and treatment:**

One to three days prior to treatment, adult rats were weighed prior to group assignment. The adult rats were assigned to groups balanced for ASR using a pretest. Juvenile groups were also ASR pretested on PND14 but matching within litters was not feasible. For adult rats, ASR Means and standard deviations were used to match groups for ASR V_{max} values. Juvenile rats were assigned to groups in the same manner on PND14 with no variation in day allowed. The use of ASR pretesting was based on preliminary data showing that body weight was not significantly correlated to ASR whereas ASR is correlated with itself across days with repeated testing. Therefore, this approach was used to reduce error variance.

The vehicle and test substance formulations were administered once by gavage 2 h prior to ASR assessment. Doses were administered to the adult males via stainless steel gavage needles with ball-tips (22-gauge). In juvenile rats, a flexible small gauge gavage needle was used. The dose volume for all groups was 5 mL/kg. Individual doses were based on the body weight recorded on the day of dose administration. Brain and plasma samples were collected to determine internal dosing. Technicians scored each animal for clinical observations prior to placing animals into the SR-LAB apparatus for ASR assessment in the juvenile experiment. This was not the case in the adult experiment; however, during the experiment, incidences of clinical observation data were collected but only after rats were placed in the SR-LAB apparatus. Test intervals were 2, 4, 6, and 8 h post-treatment. Hence, juvenile rats were rated 8 times for clinical observations and adult rats 4 times.

Adults (15 males per group) and PND15 (16/sex/group) rats were given doses of 0, 60, 90 and 120 mg/kg in 5 mL/kg corn oil. Detailed clinical observations and acoustic startle response was measured at 2, 4, 6, and 8 hrs for both age groups. Severity scoring on clinical observations was only conducted for PND 15 rats.

TABLE 1. Study design

Experimental parameter	Dose group (mg/kg bw)			
	Control	Low dose	Mid dose	High dose
Total number of animals/sex/group				
Adult Behavioral testing (Detailed Clinical Observations and Acoustic Startle)	15/Male	15/Male	15/Male	15/Male
Juvenile (PND 15) Behavioral testing (Detailed Clinical Observations and Acoustic Startle)	16/sex	16/sex	16/sex	16/sex

4. **Test Substance preparation and analysis**: The vehicle was placed in a vial for administration to the control group and for preparation of the test substance formulations. For each preparation, the test substance was warmed in a lab oven to 40 to 50˚C if it had crystallized while in storage to return it to a liquid state. It was then allowed to equilibrate to room temperature before being used to make dosing solutions. To make dosing solutions, permethrin was weighed on an analytical balance and placed in a vial to which the appropriate amount of corn oil was added. The test substance in vehicle was mixed

continuously for not less than 24 h before use and throughout dose administration procedures on a laboratory mixing plate with a magnetic stir bar placed in the solution.

Results:

Homogeneity analysis: Not reported

Stability analysis: Not reported

Concentration analysis: Not reported

4. **Statistics:** Data for adult rats were analyzed by mixed linear ANOVA, with factors of Treatment x Block and resulting in a 1-between (4 dose level factor) x 1-within (5 block factor) fixed effect factorial ANOVA model. Data for PND15 rats were analyzed similarly except that litter was accounted for by being in the ANOVA model as a randomized factor, and sex was included since both male and female pups were tested and the data analyzed by hours as the repeated measure factor. Analyses of the PND15 data showed that at this age males and females did not differ in their startle response, therefore, male and female data were combined for presentation. Data were analyzed using SAS statistical applications (SAS Institute, v 9.3, Cary, NC).

C. METHODS / OBSERVATIONS:

1. **Mortality and clinical observations:** Rats were checked regularly for mortality and morbidity. General observations were performed at the following times in juvenile rats: 2, 2.5, 4, 4.5, 6, 6.5, 8, and 8.5 h after treatment.

2. **Body weight:** Body weights were recorded on the day of ASR testing prior to dose administration.

3. **Neurobehavioral assessment:**

   a. **Functional Observational Battery (FOB):** Detailed clinical observations for the definitive experiments consisted only of salivation, motility and tremor. Several other detailed clinical observations were evaluated in preliminary studies and were determined to not to have significant results.

      The signs evaluated in pilot experiments were: lacrimation, salivation, ventral wetness, muscle tone, tremors when held, body temperature (subjectively determined, not by thermometer), other handling abnormalities, gait and posture, motility, tremor (visible), clonic seizures, tonic seizures, compulsive licking or biting, writhing, and tail pinch reactivity. Most of these did not generate any data or minimal non-zero ratings. These included perceived body temperature, handling abnormalities, gait, and posture. Several others were seen occasionally but showed so few signs that the data were not reliable. These included lacrimation (seldom seen), seizures (never seen), compulsive licking or biting (never seen), and writhing (never seen).

   b. **Locomotor activity:** Locomotor activity was not evaluated

   c. **Acoustic Startle:** Rats were tested for ASR starting 2 h following dose administration and retested at 4, 6, and 8 h intervals. Eight SDI SR-LAB startle test units were used. Each startle system was housed in an SR-LAB sound-attenuating chamber with house light, fan, and ceiling speaker. Within each chamber there is a base unit upon which the animal holder/detection plate is attached that has rubber feet to ensure stable contact with the floor of the test chamber. The animal holder/plate consisted of a base plate to which an acrylic cylindrical, horizontal tube was mounted. The cylindrical adult rat-scaled animal holder was mounted to the base plate and had removable sliding doors at both ends. A smaller animal holder scaled for young rats was used for the PND15 rats. The forward-facing door was open before the start of each test session and the animal's head was placed in the cylinder such that the animal walked into the holder on its own volition. The door was then closed behind it and once the tail was clear, the door was pressed downward until it snapped into place in a notch that secured it closed. The base units were positioned such that they did not touch the walls of the test chamber. The animal holder-base-plate assembly has a piezoelectric accelerometer force transducer mounted near the left-rear corner on the underside of each base plate, which detected the startle response as a deflection of the base plate. The ASR was measured as voltage change in fractions of a millisecond using an upgraded 6036E analogue-to-digital conversion board in the computer that is a 16 rather than the typical 12-bit board. This allows greater detection sensitivity for PND15 rats. The response recording window is 100 ms from onset of the startle stimulus. To account for non-startle movements coincident with the startle stimulus, ms-03 is used as V_{start}. Although typically zero, if this value differs from zero it is subtracted from V_{max} to account for any sudden movement of the animal prior to the onset of the startle stimulus. The peak response within the 100 ms recording window is designated as V_{max} and is measured in mV. Average response amplitude over the 100 ms response window is designated V_{avg}. As noted elsewhere, these two values are highly correlated (Pearson correlation coefficients, r =0.96-0.98), hence these two measures provide essentially identical information, therefore, we used T_{max}. The apparatus also records T_{max}, which is the latency between startle stimulus onset and V_{max}. The measure is collected automatically and the data saved but was not the focus of these experiments and is not presented in this report but can be retrieved if needed. Each test session consisted of a 5 min acclimation period in the test apparatus prior to the onset of the first acoustic stimulus. Following the 5 min no-stimulus acclimation period, 100 acoustic startle stimulus trials were presented with 20 s inter-trial intervals.

6. **Sacrifice and pathology**: N/A

7. **Positive controls:** N/A

II. DEFINITIVE STUDY RESULTS:

ADULT ANIMALS

A. OBSERVATIONS:

1. **Clinical signs:** No clinical signs were observed in animals receiving 60 mg/kg/day. At 90 mg/kg/day, 2 animals were reported with increased salivation after 4 h. At 120 mg/kg/day, 1 animal was reported with increased salivation after 4 h and 3 animals were reported to have tremors following 6 and 8 hours.

2. **Mortality:** No mortality was observed.

B. <u>BODY WEIGHT AND BODY WEIGHT GAIN</u>: No changes on bodyweight were reported.

C. <u>ACOUSTIC STARTLE RESPONSE:</u> Permethrin increased the ASR of rats in all treated groups at 4- hours and beyond. Consistent with preliminary data, 6-h post exposure was the time-to-peak effect with increases of 3.4-fold and 3-fold at the mid- and high-doses, respectively. Consistent with juvenile preliminary data, doses resulting in tremors also resulted in a diminished effect on acoustic startle. A clear dose-dependent effect was demonstrated up to 90 mg/kg/day. However, rats given 120 mg/kg did not show an increased ASR when compared to rats given 90 mg/kg/day.

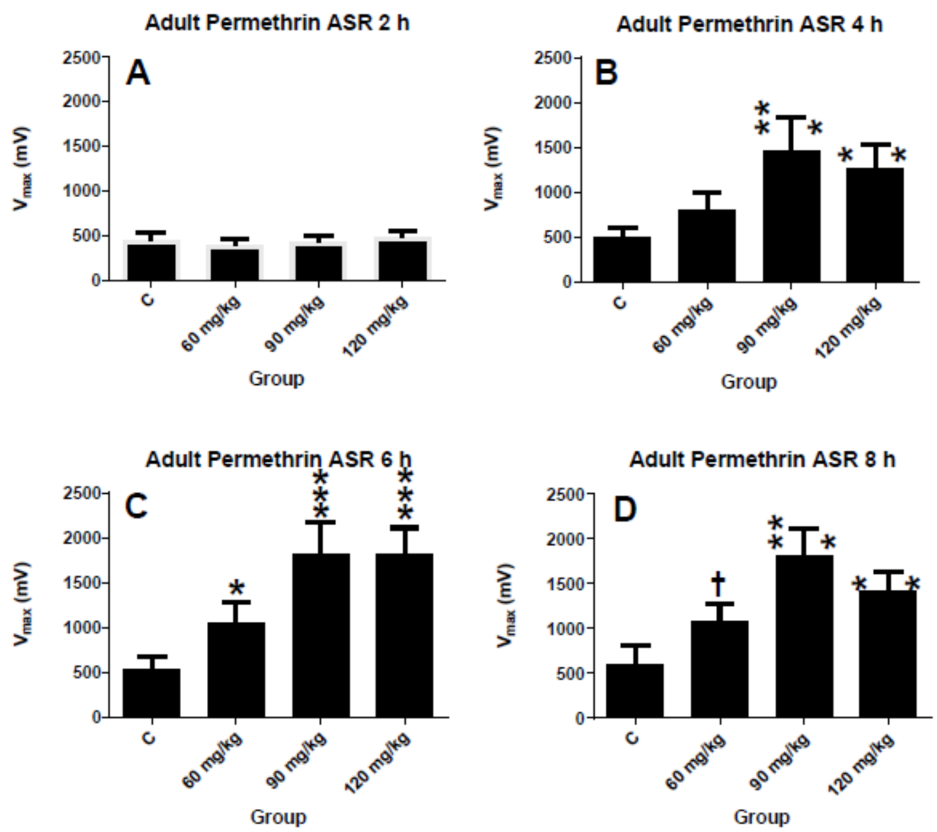

Figure 2. Adult ASR after permethrin. Increases in ASR were significant at 4, 6, and 8 h. *P<0.05; **P<0.01; ***P<0.001. (MRID 50409302; Figure 4, page 41).

JUVENILE PND 15 ANIMALS

A. <u>OBSERVATIONS</u>:

1. **<u>Clinical signs</u>:** At 60 mg/kg, mild (9/16 females; 7/16 males) to moderate (5/16 females; 7/16 males) effects on motility and mild (5/16 females; 6/16 males) to moderate (11/16 females; 10/16 males) tremors were observed from 2 to 8 h post exposure. At 90 mg/kg, moderate (6/16 females and males) to severe (10/16 females and males) effects on motility, and moderate (3/16 for females; 2/16 for males) to severe (13/16 for females; 14/16 for males) tremors were observed with an apparent time -to-peak effect at 6 h. At 120 mg/kg, severe (15/15 females; 13/14 males) effects on motility, and moderate to severe tremors (15/15 for females; 16/16 for males) were observed with an apparent time-to-peak effect of around 4 to 6 h. It is important to note the scoring system used did not differentiate between hyper- or hypo-active individual; therefore, the motility scores do not provide a good

indication of difference in activity. Summary tables of the data were not provided.

2. **Mortality:** No mortality was observed.

B. **BODY WEIGHT AND BODY WEIGHT GAIN:** No changes on bodyweight were reported.

C. **ACOUSTIC STARTLE RESPONSE (ASR):** Summary tables were not provided for acoustic startle data. In pups, a dose-dependent increase in ASR was only observed at 2 h following administration. However, the magnitude of increase at 2 h following exposure at 120 mg/kg was less than the 60 mg/kg at 6 h following exposure suggesting that increased tremor observed in pups at all dose levels impacted the acoustic startle results (Figure 3).

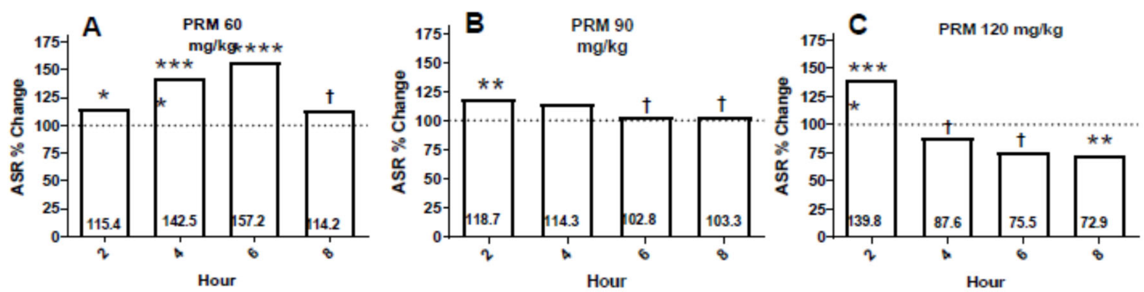

Figure 3. Percent change in the ASR after permethrin exposure on PND 15. (MRID 50409302; Figure 9, page 50).

The impact of tremor on the acoustic startle data was further supported when a comparison of ASR and tremor severity was conducted (Figure 4).

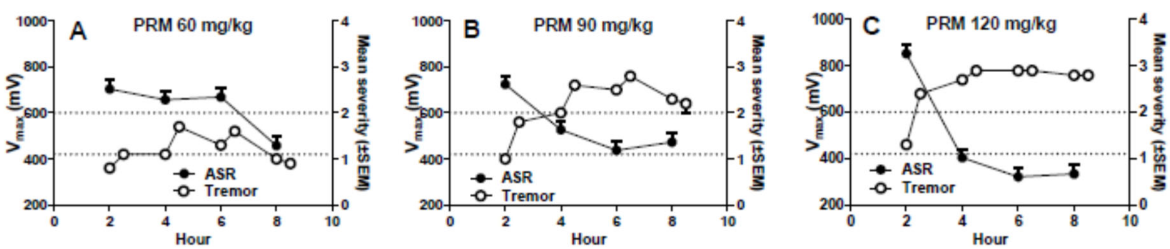

Figure 4. Permethrin-induced ASR (left axis) vs. tremor (right axis) effects in rats at PND15. (MRID 50409302; Figure 10, page 51).

As shown in Figure 4, there is a direct inverse relationship between ASR and tremors in pups. Based on the available data, it is concluded that the ASR response data in pups was conducted at too high of a dose and are not useful as a comparison with adult animals.

III. DISCUSSION AND CONCLUSIONS:

A. **INVESTIGATORS' CONCLUSIONS:** The experimental design in these experiments is well suited to demonstrate the direction, magnitude, and time-course of effects of pyrethroids on acoustic startle response at multiple dose levels in PND15 and adult rats.

The results establish that ASR is an effective, quantitative method to measure acute neurotoxicity of permethrin in adult and PND15 rats and demonstrate not only the sensitivity of ASR but its reliability based on the reproducibility of effects between preliminary and definitive experiments. The data firmly establish that PND15 rats are more sensitive to permethrin than are adult rats. The experiments also confirmed that PND15 rats are also more sensitive to the neurological (DCO) symptoms induced by permethrin than are adults. The high dose in PND15 rats showed severe tremor, consistent with the "T" syndrome associated with Type I pyrethroids.

B. REVIEWER COMMENTS The study supports that treatment with permethrin increased the ASR in PND15 rats. However, the available data did not demonstrate an appropriate magnitude or time-course of effects on acoustic startle in PND15 rats. In both preliminary and definitive studies, the available data indicate that tremors interfered or confounded the results of the acoustic startle testing in PND15 rats. Based on the available data, it is not clear to the reviewer why dosing in PND15 pups was conducted at the same level as adults for the definitive experiments. PND15 pups were clearly more sensitive throughout the preliminary testing. Due to the poor quality of data in PND15 pups a quantitative comparison between juveniles and adults is not appropriate using ASR data in this study. Additionally, the detailed clinical examinations could not be compared between juveniles and adults since limited observations were conducted in the adults.

Appropriate doses for PND15 pups were not established, which in turn did not allow study authors to establish a clear time to peak effect, or post-treatment interval for testing. The dose selection for the definitive study was not reviewed by the Agency prior to the initiation of the study; the Agency would have preferred to participate in the dose selection process. The reviewer does agree that PND15 pups were clearly more sensitive to the neurotoxic effects of permethrin based on clinical observations.

Appendix A

Preliminary Data Tables and Figures

Table 1A: Permethrin 120 mg/kg by gavage in 1 mL/kg corn oil on ASR in adult LE male rats. (From MRID 50409302; Table 9, page 34).

		Mean ± SEM		
Group	time (h)	N	Mean	SEM
Control	2	5	143.98	52.12
Permethrin	2	5	1392.03	559.92*
Control	4	5	159.19	40.86
Permethrin	4	5	1782.03	534.22*

*P < 0.05

Table 2A: Permethrin 120 mg/kg by gavage in 1 mL/kg corn oil on ASR in adult SD male rats. Mean±SEM. (From MRID 50409302; Table 11, page 35).

Group	time (h)	N	Mean	SEM
Control	2	4	383.99	121.31
Permethrin	2	6	1094.84	246.88*
Control	4	4	316.54	103.81
Permethrin	4	6	2337.47	388.04*

*P < 0.05

Table 3A: Permethrin 120 mg/kg by gavage in 5 mL/kg corn oil on ASR in adult SD male rats. Mean ± SEM (From MRID 50409302; Table 13, page 36).

Group	time (h)	N	Mean	SEM
Control	2	6	327.72	49.94
Permethrin	2	6	334.24	49.94
Control	4	6	299.39	105.61
Permethrin	4	6	729.42	105.61
Control	6	6	284.13	270.88
Permethrin	6	6	909.53	270.88

Table 4A: Time since treatment (ordinary Means ± SEM). (From MRID 50409302; Table 15, page 38).

2 h			Mean	SEM	Fold change
	treat	C	312.05	62.46	
	treat	P	557.55	122.42	1.8 times higher
4 h					
	treat	C	325.63	33.49	
	treat	P	1436.77	503.36	4.4 times higher
6 h					
	treat	C	294.10	37.38	
	treat	P	2079.72	1001.17	7.1 times higher
8 h					
	treat	C	312.12	51.08	
	treat	P	1492.99	485.80	4.8 times higher

Note: C = Control (corn oil); P = PRM (120 mg/kg)

Table 5A: Mortality following Permethrin Treatment in Juvenile Rats. (From MRID 50409302; Table 1, page 19).

Table 1 PRM-induced Mortality		
Dose (mg/kg)	Age	
	Number	%
	PND15	
120	0/20	0
150	0/16	0
180	2/15	13.3
	PND17	
120	0/18	0
150	0/21	0
180	1/17	5.9
	PND21	
120	0/15	0
150	0/12	0
180	6/14	42.9%

Table 6A: Incidence of Tremor following Permethrin Treatment in Juvenile Rats. (From MRID 50409302; Table 2, page 21).

Table 2
PRM-induced Tremor

Age PND	Hour (post-treatment)	Dose (mg/kg) (in 5 mL/kg corn oil)		
		120	150	180
	Actual dose	72	90	108
15	2	4/20 (20%)	13/16 (81.2%)	15/15 (100%)
	4	15/20 (75%)	13/16 (81.2%)	15/15 (100%)
	6	7/20 (35%)	8/16 (50%)	15/15 (100%)
	8	4/20 (20%)	1/16 (6.2%)	11/13 (84.6%)
17	2	2/18 (11.1%)	17/21 (81.0%)	14/17 (82.4%)
	4	8/18 (44.4%)	20/21 (95.2%)	17/17 (100%)
	6	4/18 (22.2%)	18/21 (85.7%)	15/17 (88.2%)
	8	1/18 (5.6%)	12/21 (57.1%)	10/16 (62.5%)
21	2	3/15 (20%)	9/12 (75%)	14/14 (100%)
	4	1/15 (6.7%)	11/12 (91.7%)	11/11 (100%)
	6	0/15 (0%)	6/12 (50%)	8/8 (100%)
	8	0/15 (0%)	0/12 (0%)	1/8 (12.5%)

Table 7A: Effects of PRM on ASR: Preliminary Dose-range finding experiment. (From MRID 50409302; Table 3, page 23).

Age[1]	Dose[2]	Grp	2 h	4 h	6 h	8 h	# Litters	# Pups
15	72	Con	566.8 (53.8)	410.0 (27.3)	358.9 (31.8)	357.2 (44.0)	5	16
		PRM	571.7 (48.1) [+0.8%]	477.9 (24.4)* [+16.5%]	418.4 (28.5) [+16.5%]	375.4 (39.3) [+5%]		20
	90	Con	444.6 (59.8)	387.9 (41.6)	431.6 (40.4)	419.8 (30.2)	5	14
		PRM	897.0 (55.9)# [+101%]	562.2 (38.9)# [+44.9%]	511.7 (37.8)# [+18.5%]	443.9 (28.2) [+5.7%]		16
	108	Con	407.7 (47.8)	301.9 (39.3)	283.2 (36.1)	267.6 (21.8)	5	13
		PRM	683.2 (44.5)# [+67.6%]	421.4 (36.6)# [+39.6%]	355.3 (33.6) [+25.5%]	300.4 (21.8) [12.3%]		15
17	72	Con	557.2 (67.6)	416.6 (55.1)	445.9 (54.4)	430.8 (35.7)	5	15
		PRM	690.3 (69.6) [+23.9%]	627.1 (56.8)# [+50.5%]	488.2 (56.1) [+9.4%]	381.6 (36.8) --		18
	90	Con	624.1 (84.9)	410.5 (51.7)	409.7 (52.5)	413.2 (49.6)	5	16
		PRM	979.7 (74.1)# [+60%]	601.2 (45.2)# [+46.5%]	574.3 (45.8)# [+40.2%]	503.3 (43.3) [+21.8%]		21
	108	Con	430.0 (83.6)	360.3 (80.4)	329.7 (65.7)	355.4 (50.0)	5	13
		PRM	629.1 (73.1)* [+46.3%]	563.6 (70.3)* [+56.4%]	493.4 (57.4)* [+49.7]	437.1 (45.1) [+23%]		17
21	72	Con	812.2 (100.9)	661.4 (75.8)	598.3 (76.1)	355.4 (50.0)	5	14
		PRM	1040.1 (97.5) [+28.1%]	857.5 (73.3)* [+29.6%]	682.3 (73.5) [+14%]	437.1 (45.1) [+23%]		15
	90	Con	840.0 (73.5)	729.7 (85.3)	574.4 (53.0)	590.3 (79.8)	4	12
		PRM	1453.1 (73.5)# [+73%]	1220.0 (85.3)# [+67.2%]	993.0 (53.0)# [+72.9%]	818.4 (79.8)‡ [+38.6%]		12
	108	Con	679.2 (65.6)	634.0 (56.0)	605.5 (48.7)	635.7 (254.7)	5	13
		PRM	1148.5 (63.2)* [+69.3%]	1224.4 (60.9)* [+93.1%]	917.8 (57.1)* [+51.6%]	1319.3 (298.7)* [+107.5%]		14

[1]PND = postnatal day; [2]Dose expressed as mg/kg (corrected).

*P<0.05 one-tailed, #P<0.05 two-tailed; ‡Treatment x trial block interaction (Mean ± SEM)